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Quantitation of Pyrethrum Residues in Brown Tree Snakes

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Abstract

A reversed-phase solid-phase extraction–gas chromatography (SPE–GC)–electron capture detection method is developed to quantitate individual rethrin residues in pyrethrum-exposed brown tree snakes. Aliquots (6 g) of homogenized snake tissue are extracted with 10 mL acetonitrile. The rethrins are recovered from the acetonitrile extract and concentrated using C_8 SPE. The rethrins are eluted from the SPE column with pentane, evaporated to near dryness, and reconstituted to 1 mL with 1-propanol. Individual rethrins are quantitated using GC analysis of the 1-propanol solution. Method limits of detection for rethrins range from 0.63 to 6.51 ng/g. The mean recovery for all rethrins is 70.8% with a standard deviation of 5.7%. This method is used to successfully quantitate incurred rethrin residues in pyrethrum-exposed brown tree snakes.

Introduction

The brown tree snake (*Bioga irregularis)* is a significant pest that was probably introduced to Guam via cargo from Papua New Guinea after World War II (1). Since its initial detection in Guam in the 1950s, the brown tree snake has increased its range throughout the island with recorded population densities as high as several thousand per square mile. The brown tree snake is arboreal and nocturnal. It is generally accepted that the brown tree snake is the primary cause of elimination of much of the avian life in Guam (2). Several avian species are extinct, and several others are threatened. Brown tree snakes are agricultural pests because they kill chickens, pigeons, and newborn pigs (3).

Brown tree snakes are also nonagricultural pests because they cause frequent power outages by climbing power poles, simultaneously touching live and grounded conductors, and short circuiting electric transformers. Between 1978 and 1988, 562 such power outages were reported in Guam, resulting in losses of millions of dollars (4).

The brown tree snake is also a public health threat. Between August 1989 and October 1990, there were 49 documented brown tree snake bites in Guam; 60% of the victims were children under 6 years old. Because the brown tree snake is mildly venomous, attacks have resulted in severe reactions, but no deaths have been documented (5).

Because Guam is a major transfer point for air and ship cargo traffic in the Pacific, there is a threat that the brown tree snake could inadvertently be introduced to other Pacific islands via cargo shipments. Although brown tree snakes have been discovered on the Hawaiian Islands, Wake Island, Kwajalein Island, and Saipan, Guam has the only known reproductive population outside of the snake's native range (3).

As part of a multi-agency program, the U.S. Department of Agriculture (USDA)/Animal Plant Health Inspection Service/Wildlife Services/National Wildlife Research Center (NWRC) has been developing and evaluating methods to mitigate and control brown tree snakes. A major area of research involves the identification of potential brown tree snake toxicants. Because anecdotal evidence suggested that pyrethrum/pyrethrins insecticide formulations have inadvertently resulted in pet snake mortality (6), an evaluation of natural pyrethrum extracts and the synthetic pyrethroids allethrin, permethrin, phenothrin, tetramethrin, and resmethrin for dermal and oral brown tree snake toxicity was made (7). Commercially available pyrethrum and pyrethroid foggers were also evaluated as potential cargo fumigants for brown tree snake control (8). Orally, pyrethrum was the most toxic compound for brown tree snakes. Among dermal applications, both allethrins and pyrethrum exhibited the greatest toxicity. These results suggested that pyrethrum has excellent potential for brown tree snake control. To evaluate the potential for secondary hazards caused by predator consumption of brown tree snakes containing pyrethrum residues, a method for quantitating pyrethrum in brown tree snakes was needed. Also, the quantitation of residues in brown tree snakes could provide valuable information to explain the differences in toxicity associated with various carriers. Because the literature is void of residue methods for pesticides in snakes, the required analytical methodology needed to be developed.

Pyrethrum is a botanical insecticide extracted from the dried flowers of *Chrysanthemum cinerariaefolium*. The insecticidal compounds are the rethrins: pyrethrins I and II (PI and PII),

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cinerins I and II (CI and CII), and jasmolins I and II (JI and JII) (Figure 1). Compounds with the I designation are esters of chrysanthemic acid, and compounds with the II designation are esters of pyrethric acid. For pyrethrins, cinerins, and jasmolins, esters of the acids are formed with pyrethrolone, cinerolone, and jasmolone, respectively (9). Quantitation of these compounds in the refined plant extract is primarily via the AOAC/titrimetric procedure. This method, which has been accepted both by industry and government regulators, quantitates the active ingredients as pyrethrins I and II. Unfortunately, this method is not acceptable for the analysis of residue levels of individual rethrins or for pyrethrum in mixtures with other active ingredients (10).

Separation of the 6 individual rethrins has been accomplished by both gas chromatography (GC) and high-performance liquid chromatography (HPLC). The conjugation of the ketone to the double bond in the cyclopentanone ring offers fairly good detection by electron capture detection (ECD) following GC separation. This approach has been successfully used to quantitate the individual rethrins in refined extracts (11–20). Some researchers have used HPLC with ultraviolet (UV) detection to quantitate rethrins (21–25). However, because of the relatively limited absorbtivity of the chromophore in the rethrins, the sensitivity of UV detection coupled with HPLC is less than that which can be obtained with GC–ECD. Furthermore, GC–ECD proved to be more selective than HPLC–UV for the analysis of pyrethrum extracts of biological matrices. To determine rethrin residues in brown tree snakes, methodology based on GC–ECD was developed.

Experimental

Chemicals and materials

Pyrethrum concentrate (45%) was obtained from MGK (Chaska, MN). HPLC-grade methanol, 1-propanol, ethyl acetate, *n*-pentane, and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ). HPLC-grade hexane was from J.T. Baker (Phillipsburg, NJ). Octane, methyl octanoate, 1,6-heptadiene, 1-heptene, and 1-hexene were obtained from Aldrich (Milwaukee, WI). Solid-phase extraction (SPE) cartridges (J.T. Baker) containing 1 g C_8 sorbent in 6-mL cartridges were used with 75-mL auxiliary reservoirs. Frozen snakes were homogenized in a stainless steel tissue pulverizer (20 cm × 7.9-cm i.d.) (26).

Standard solutions

Concentrated stock solutions were prepared by diluting 40 or 800 mg of the 45% pyrethrum concentrate (26.19% PI and 19.32% PII) to 10 mL with ethyl acetate to produce concentrated stock solutions A and B, respectively. Intermediate stock solutions A_1 and A_2 were prepared by transferring 1 mL or 50 µL, respectively, of concentrated standard solution A to a 10-mL volumetric flask and diluting to volume with ethyl acetate.

The concentration of each rethrin in the concentrated solutions was calculated from the results of the GC analyses as follows:

(peak area for individual rethrin/total peak area for class [I or II] × 100% × stated purity for class (I or II) = percent of individual rethrin Eq 1

(weight of concentrate/10 mL) × percent of individual rethrin = concentration of individual rethrin Eq 2

As indicated in Table I, stock solutions were diluted to prepare calibration standards for linearity. Appropriate dilution factors were applied to calculate the concentration of individual rethrins in calibration standards.

Samples

Brown tree snakes were captured in Guam. Snakes exposed to pyrethrum and non-exposed controls were euthanized, immediately frozen, and kept frozen during storage, shipment to our laboratory, and subsequent storage and homogenization. Control snakes were analyzed for the absence of chromatographic interferrants or were fortified with pyrethrum extract for method development and quality control. Control snakes were fortified at 0.08, 2.5, and 90 µg pyrethrum/g tissue by adding 50 µL intermediate standard solution A_2, 80 µL intermediate standard solution A_1, or 15 µL concentrated standard solution B, respectively, to 6 g of homogenized snake tissue. Snakes exposed to pyrethrum during toxicity testing in Guam were analyzed for incurred pyrethrum and individual rethrin residues.

Sample preparation

Frozen brown tree snakes were chopped into sections approximately 5–10 cm long, and an aliquot of up to 100 g of tissue was placed in the pulverizing device. To further lower the temperature of the tissue, liquid nitrogen was added until vigorous boiling stopped. The frozen tissue was pounded repeatedly (protective insulated gloves and eye protection were employed) until the tissue was homogenized into a pourable powder (26). Aliquots (6.0 g) of the snake tissue were transferred to a 50-mL screw-top glass centrifuge tube. To extract rethrins from tissue, 5.0 mL acetonitrile was added to each tube. The tubes were capped and shaken on a horizontal shaker for 10 min. The tubes were sonicated for 10 min and centrifuged at 75 × *g* for 2 min. The acetonitrile supernatant was carefully decanted into a 250-mL Erlenmeyer flask containing 225 mL deionized water. The tissue extraction was repeated one more time with the acetonitrile added to the same flask. The acetonitrile–water solution was mixed and eluted through J.T. Baker C_8 SPE cartridges that had been preconditioned with 10 mL methanol and 5 mL deionized water. Centrifugal force from a benchtop centrifuge was used to facilitate elution. After the entire sample extract eluted through the column, the columns were dried by drawing air through them for several minutes. Rethrins were recovered from the SPE cartridge with duplicate elutions of 5 mL *n*-pentane. The rethrins containing pentane eluent were combined in a volume-calibrated 10-mL centrifuge tube and evaporated to near dryness at room temperature under a gentle stream of nitrogen. The sample was reconstituted to 1.0 mL with 1-propanol. After mixing on a vortex, the reconstituted extract was transferred to a GC autosampler vial, capped, and loaded into the autosampler for GC analysis.

Instrumentation

Aliquots (1 µL) of snake extracts were analyzed on a Hewlett-Packard (Wilmington, DE) 5890 GC equipped with an ECD. Separation was accomplished on a DB-5 (J&W Scientific, Folsom,

CA) capillary column (30 m × 0.32-mm i.d., 0.25-µm film thickness). The oven temperature was programmed with an initial temperature of 90°C for 0.5 min, followed by a 25°C/min ramp to 210°C, a 210°C 29-min isothermal period, a 60°C/min ramp to 295°C, and a final 295°C 6.5-min isothermal bake out, used to quantitate the individual rethrins. A flow rate of 4 mL/min was used for the helium carrier gas and the argon–10% methane detector make-up gas. The ECD and injector port temperatures were 300°C. To condition the injector and GC column, several extracts of pyrethrum-containing snake extracts were injected prior to sample analyses and after every 10 injections of standards. An oven temperature of 50°C was used to determine the relative ECD response factors (peak area/mass) for octane, methyl octanoate, 1-hexene, 1-heptene, and 1,6 heptadiene.

GC–mass spectrometry (GC–MS) was accomplished using a Hewlett-Packard 5890 GC and 5972 mass selective detector. GC conditions were as previously noted, except that a DB-5 (30 m × 0.25-mm i.d., 0.25-µm film thickness) column was used. MS conditions were as follows: ionization energy, 70 eV; mass range, *m/z* 50–400; ion source temperature, 280°C. Chromatographic peaks for rethrins were identified by comparing mass spectra with the National Bureau of Standards library.

Validation

Because the range of anticipated residues varied by more than 1000, linearity was determined with the 3 sets of standards listed in Table I. Because of the limited linear range of the GC–ECD under the stated operating conditions, each set of standards covered a concentration range of approximately 20. Each standard solution was analyzed in duplicate using GC. Linearity was determined using linear regression analysis of mean response versus concentration for each set of standards. Resulting regression equations from the appropriate curves were used to determine the concentrations of individual rethrins in brown tree snakes. Analysis of extracts from pyrethrum-fortified snakes was used to determine the recovery efficiency of the analytical method. The analysis of extracts from control snakes confirmed the lack of chromatographic interferants at the retention times of the rethrins. Chromatograms of extracts from the control and the snakes fortified at low level were used to determine the method limit of detection (MLOD). MLODs were calculated for each rethrin on each analysis day as the concentration of analyte that would produce a chromatographic peak response equal to 3 times the chromatographic response at the same retention time in the control matrix.

Results and Discussion

In an attempt to minimize the thermal degradation of rethrins during GC analysis, it was hoped that oven temperatures could be kept to a minimum by using capillary GC columns of 15 m or less (11). Initial efforts proved unsuccessful, because DB-1, DB-5, and DB-17 columns of this length did not provide an adequate resolution to separate the analytes of interest from co-extracted matrix components. Retention times for all 6 rethrins and the isopyrethrins are listed in Table II. Under the chromatographic conditions used, approximately 50–75% of PI and PII were detected as their thermal isomerization products iso-PI and iso-PII (Figure

Table I. Preparation of Calibration Standards

		Standard	Final concentration (µg/mL)*					
	Volume	solution	CI	JI	PI	CII	JII	PII
Low								
	10	A_1	0.0425	0.0209	0.0423	0.0498	0.0197	0.00847
	25	A_1	0.107	0.053	0.106	0.124	0.495	0.0209
	50	A_1	0.212	0.105	0.211	0.249	0.0981	0.0419
	100	A_1	0.426	0.209	0.423	0.498	0.197	0.0857
	200	A_1	0.851	0.417	0.846	0.996	0.394	0.170
Medium								
	250	A_1	1.06	0.52	1.05	1.25	0.49	0.210
	50	A	2.21	1.05	2.11	2.5	0.98	0.419
	100	A	4.26	2.09	4.23	1.90	1.97	0.848
	250	A	10.6	5.22	10.6	12.4	4.92	2.11
	500	A	21.2	10.5	21.1	24.95	9.81	4.24
High								
	1000	A	42.6	20.9	44.4	49.8	19.7	8.9
	75	B	60.2	29.5	59.9	70.5	27.9	12.0
	150	B	120	59.0	120	140.9	55.8	24.0
	300	B	240	118.1	240	282	111	47.9
	500	B	402	197.1	399	470	186	79.9
	1000	B	802	393	798	940	372	160

* Based on 40 mg pyrethrum concentrate in 10 mL ethyl acetate for concentrated stock solution A.

Table II. GC Retention Times

Rethrin	Retention time (min)
CI	11.1
JI	12.9
PI	13.7
iso-PI	17.5
CII	23.2
JII	28.4
PII	30.7
iso-PII	35.1

Table III. Method Limits of Detection

Rethrin	Mean MLOD (ppb)	MLOD range (ppb)
CI	2.83	1.29–5.76
JI	3.03	1.34–4.35
PI	3.11	0.63–4.87
CII	1.75	0.63–3.22
JII	3.30	1.46–4.40
PII	5.31	3.32–6.51

Table IV. Rethrin Recoveries from Fortified Brown Tree Snakes*

Compound	µg/g†	Rec%‡	µg/g	Rec%	µg/g	Rec%
CI	0.038	55.3	1.0	65.9	35	79.0
JI	0.018	48.1	0.51	56.1	17	66.0
PI	0.038	64.1	1.0	66.4	35	67.9
CII	0.045	93.5	1.2	81.9	41	79.0
JII	0.017	85.4	0.48	70.8	16	75.3
PII	0.008	115.6	0.21	73.5	7	74.1
Total Rethrins	0.163	77.0	4.4	69.1	151	73.5

* Mean of 3 snakes.
† Rethrin fortification concentration.
‡ Percent recovery.

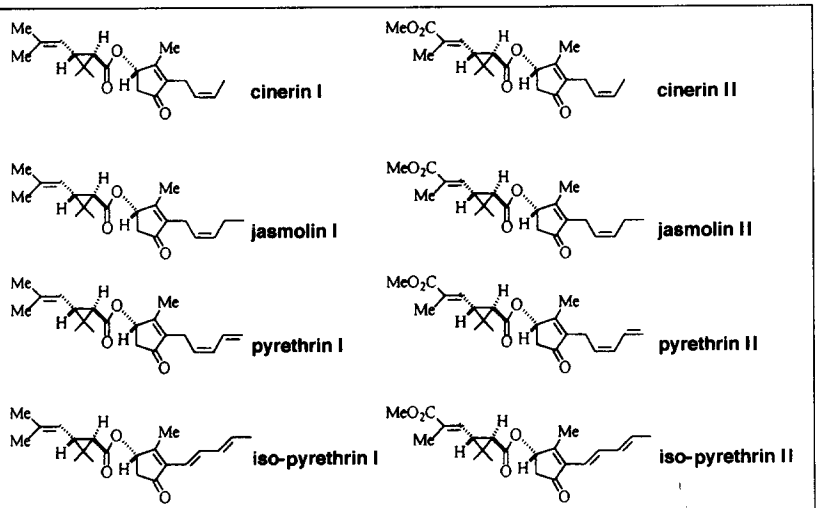

Figure 1. Biologically active rethrin constituents of pyrethrum and thermally induced isomers of pyrethrins.

1). The areas of the chromatographic peaks corresponding to the pyrethrins and iso-pyrethrins were combined to determine the total concentration of each pyrethrin in the sample. In a separate experiment, manipulation of the oven temperature in an attempt to produce different ratios of iso-P/P had little effect on the combined peak areas.

For the calculation of rethrin concentrations, it was assumed that response factors were identical for the 3 rethrins in each class. The ECD response to rethrins is primarily caused by the conjugation of the ketone to the double bond in the cyclopentone ring (Figure 1). This portion of the molecule is consistent for all 6 rethrins. On the acidic portion of the molecule, type II rethrins contain a terminal ester rather than the methyl moiety found in type I rethrins. The ECD response factor for the ester-containing compound methyl octanoate was approximately more than 100 times greater than that observed for octane, suggesting that the methyl ester in type II rethrins contributed more to the ECD response than the corresponding methyl in type I rethrins. This indicates that the response factors for type I and type II rethrins must be calculated separately. Jasmolin, cinerin, and pyrethrin differ with respect to the alkene attached to the cyclopentanone portion of the molecule (Figure 1). ECD response factors for the analogous series of alkenes, 1-hexene, 1-heptene, and 1,6-heptadiene were nearly identical and were more than 1000 times less than the response factors observed for the rethrins. This suggests that assuming identical ECD response factors for the 3 rethrins within each class was also valid. Furthermore, because the alkene portion of PI and PII were isomerized to form iso-PI and iso-PII, these data also indicate the validity of combining the peak areas for the isomerized and the parent compound to determine the pyrethrin concentrations in the original samples.

The response factors for each rethrin decreased as the concentration of the standards increased. Because of the limited linear range of the ECD, it was necessary to generate curves for 3 separate concentration ranges. For each of the 3 linearity data sets, regression analyses for each compound resulted in correlation coefficients of 0.95–0.99.

MLODs (Table III) were determined from chromatograms of control and low-level fortified samples. MLODs ranged from 0.630 ng/g for CII to 6.51 ng/g for PII. The recoveries of rethrins from fortified control brown tree snakes are shown in Table IV. Fortification levels of total rethrins varied by approximately 1000, ranging from 0.163 to 151 µg/g. Although the mean recovery for total rethrins was quite consistent, recoveries for individual rethrins were variable at the lowest fortification level. Recoveries of individual rethrins ranged from 48.1% for JI at 0.018 µg/g to 115.6% for PII at 0.008 µg/g. The overall mean recovery for each rethrin at all levels was 70.8% with a standard deviation of 5.7%.

This method was used to quantitate rethrins in brown tree snakes that were exposed to pyrethrum during toxicity testing in Guam. Residue results are presented in Table V. Snakes were treated dermally with 1% pyrethrum extract in ethanol for 1 h. After 3 days, 100% mortality was observed. In other experiments, unrestrained

snakes crawled through the 1% pyrethrum–soybean oil. In this experiment, the absorbed dose varied presumably as the time that the snake was in contact with the toxicant solution varied. Mortality of the pyrethrum-exposed snakes in these experiments ranged from 50 to 88%. The mean total pyrethrums in surviving snakes was 4.5 µg/g, and the mean total pyrethrum concentration in snakes receiving a lethal dose was 18.1 µg/g. Rethrin residues in orally treated snakes were significantly higher than those in dermally treated snakes. Snakes were orally dosed with 20 or 40 mg pyrethrum per snake. For snakes receiving 20 mg, surviving snakes contained mean residues of 66.4 µg/g, and fatally dosed snakes contained mean residues of 122.8 µg/g. For snakes receiving an oral dose of 40 mg pyrethrum, the mean residues in surviving and fatally dosed snakes were 16.7 and 306.9 µg/g, respectively. The mean residue in fatally dosed snakes was 188 mg/kg as compared to 100 mg/kg for surviving snakes.

This method proved to be sufficiently rugged for the analysis of pyrethrum residues in snakes from 4 different toxicity studies conducted in Guam. The resulting data are currently being used to develop management strategies aimed at reducing the brown tree snake population. This will hopefully be the first step in restoring the perturbed ecological balance of this Pacific island and preventing similar disasters from occurring elsewhere.

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Table V. Mean Residues in Pyrethrum Treated Snakes*

	CI	JI	PI	CII	JII	PII	Total
Dermal							
1% pyrethrum							
survived	0.95	1.01	1.58	0.34	0.49	0.15	4.5
died	2.66	3.4	5.12	2.25	3.96	0.69	18.1
Oral							
20 mg							
survived	14.5	13.0	18.2	9.48	7.58	3.65	66.4
died	24.8	21.5	32.2	23.3	13.8	7.24	122.8
40 mg							
survived	3.72	3.18	3.93	3.33	1.86	0.70	16.74
died	60.25	50.6	74.0	72.3	33.05	16.68	306.9

* Observed micrograms of rethrin per gram of snake.

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